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PATENT GAZETTE

OIL BODY-LIKE OR SHELL-LIKE NANOCAPSULES AND THE PREPARATION

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Assignee: Nippon Suisan Kabushiki Kaisha (000004189), #6-2 2 Chome, Ohte-cho, Chiyoda-ku, Tokyo
Assignee: Kaneda Kabushiki Kaisha (595064854), #34-9 Asakusabashi 1 Chome, Taito-ku, Tokyo
Assignee: Meiji Seika Kabushiki Kaisha (000006091), #4-16 Kyobashi 2 Chome, Chuo-ku, Tokyo
Inventors: Tomotada Ono, Iwate University, #18-34 Ueda 3 Chome, Morioka, Iwate Prefecture
Tomonori Tsukamoto, Twate University, #18-34 Ueda 3 Chome, Morioka, Iwate Prefecture
Patent Attorney: Asami Sudo

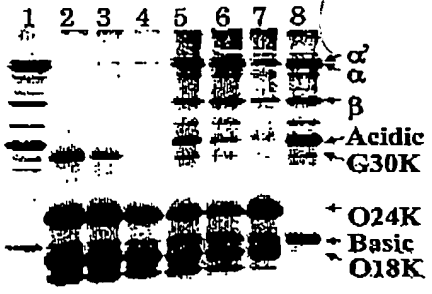
Title of the Invention

Oil body-like or shell-like nanocapsules and the preparation.

Summary

Objective: To provide oilbodies in the separated and recoverable form, to provide materials for dry nanocapsule shells and to apply them in the stabilization of hydrophobic materials like lipids.

Means of solution: The oil body-like nanocapsule fraction or its dried material containing oil seeds with oleosin protein, preferably the soybean-derived, or better, oleosin protein over 10% of the total protein. Oil body nanocapsule fraction containing over 10 % of oleosin protein derived from soybeans or oleosin-containing oil seeds of the total proteins, or its dried shell-like nanocapsules wherein neutral lipids have been removed, preferably the shell-like nanocapsules for coating hydrophobic substance(s), or more specific, the shell-like nanocapsules for coating more than one lipid selcted from rapeseed oil, fat-soluble vitamins and fish oil. It does not containing at least the Gly m Bd 30K of the allergenic proteins of soybeans.



Claims

(1) Oil body-like nanocapsule fraction derived from oil seeds containing oleosin protein.
(2) In Claim (1) wherein the oleosin protein is over 10 % of the total protein.

(3) In Claim (1) or (2) wherein the said oil seeds are soybean seeds.
(4) In Claim(1), (2) or (3) wherein the dried materials are the oil body-like nanocapsule fraction.
(5) In Claim (1), (2), (3) or (4) wherein the shell-like nanocapsules are made from the said oil body-like nanocapsule fraction wherefrom the neutral lipids are removed.
(6) In Claim (5) wherein the shell-like nanocapsules are used for the inclusion of hydrophobic materials.
(7) In Claim (6) wherein the said hydrophobic materials are more than one lipds selected arbitrarily from rapeseed oil, fat-soluble vitamins and fish oil.
(8) In one of the Claims (5) through (8) wherein the shell-like nanocapsules do not contain the allergenic protein (Gly m Bd 30K).
(9) Process to prepare oil body-like nanocapsule fraction characterized using oil seeds containin oleosin protein as the starting material by soaking them in water, grinding or after grinding soaking in water to separate the insolubles and the resultant suspension is heated over 60 °C, or after heating at over 60 °C, to separate the insolubles to get a suspension to get oil body-like nancapsule fraction of high oleosin purity.
(10) In Claim (10) wherein after heating the solution is centrifuged to enhace purity of oleosin.
(11) In Claim (10) or (11) wherein neutral lipids are removed from the oil bldy-like nanocapsule fraction to make shell-like nanocapsules.
(12) In Claim (10) or (11) wherein the said oil body-like nanocapsue fraction is dried followed by removing neutral lipids to make shell-like nanocapsules.

Details of the Invention

Technical field of the invention: This invention is about the preparation of oil body-like or shell-like nanocapsules. To be more specific, it is about the preparation of the oil body-like nanocapsule fraction or the dried shell-like nanocapsules rich in oleosin which can be used to include hydrophobic substances like rapeseed oil, fat-soluble vitamins and fish oil, etc.

Previous technics: Soybean is not only rich in proteins but also contains lots of oil. In Japan soybean has been used as the important source of proteins but lately it has been used as oil seeds and it has become the primary source of vegetable oil. In Japan, it serves as the indispensable source of food like tofu, soysauce, natto and miso. Miso and soysauce get their unique flavor from degradation of proteins and lipids in soybean to peptides, amino acids, fatty acids, glycerols and their derivatives. In natto more than 60% of its proteins are degraded. Meanwhile, tofu is prepared by soaking soybeans in water to grind, and after heating the bean curd refuse is removed and either bittern or

glucono-delta-lactone (GDL) is added for coagulation. Almost all the proteins and lipids in soybean remain in the coagulate. Though depending on the kind of soybean used but almost in every case tofu contains, on dry weight basis, 55% proteins and 35% lipids. Frozen tofu is prepared by freezing tofu followed by desiccation so it contains exactly these amounts of proteins and lipids. In meat and fish oil is separated by heating while in tofu and frozen tofu the lipid portion will not separate. They are the kinds of food which do not make you feel that they contain so much lipids.

In soybean seeds lipids are accumulated in spherozomes and form oil bodies. An oil body has a structure which looks like protein tacks in neutral fats surround phospholipids and through such a structure it maintains oil droplets stably. The protein is called oleosin and in many oil seeds it has molecular weight of 15-26K and was found to have similar amino acid sequences. An oleosin consists of three domains which have the amphipathic N termini, the central portion consisting of anti-parallel beta sheet and alpha-helix and the C-terminus consisting of alpha-helix. In the case of soybean 24 and 18K oleosins are found. Here, the center consists of about 70 hydrophobic amino acids. This portion is almost the same in any oleosins and difference in molecular weights of different oleosins is known to depending on the length of the C terminus. The hydrophobic portion of the central part assume needle shape and punctures into the lipid clump while the N and the C termini are half-buried and half submerged in water.

Problems that this invention intends to solve: In unheated raw soy milk lots of 7S, 11S proteins and allergenic proteins are attached to oil bodies. Therefore, despite of containing lots of lipids the specific weight of oil body is heavy and even if raw soy milk is ultracentrifuged (156,000 x g, 30 minutes) the oil bodies will not float. Also there are many ways to separate the 7S and 11S proteins by addition of large amount of salts (e.g., NaCl, etc.), reductant or base but recovery is very poor or purity of oil bodies recovered is extremely low so these methods are not practical in the preparation of materials in the production of dried nanocapsule shells.

As mentioned above the current situation of the preparation of oil body remains such that by such conventional methods it is very difficult to separate and recover oil bodies efficiently. This invention is therefore to provide separated and recovered oil bodies, to provide materials for the preparation of dried nanocapsule shells and also to use them to stabilize hydrophobic substances like lipids. Also, the invention aims at offering dried shell-like nanocapsules of oleosin-rich body which can be used in the preparation of hydrophobic substance-including nanocapsules wherein the oil body-centered neutal lipids can be substituted by other substances. The microcapsues prepared by the convention-

al technics have particle diameter in the range of microns which can be visually confirmed of their presence. Also, when hydrophobic substances are to be included they are coated by solid lipid or polysaccharide. As the result of which the ratio of these coating materials to the inclusion increases. To solve these problems liposomes were developed which have hydrophobic substance coated by phospholipid. A liposome has a diameter ranging from two to three digit microns. Its coating membrane is also very thin so it seems to have solved the said problems but a liposome is very heat-unstable and besides, during storage they tend to gradually coalesce to large particles so they pose problems in emulsification stability. This invention is to offer fine capsules superb in heat and emulsification stability.

Means to solve the problems: An oil body is an intracellular structure consisting of proteins like oleosins and phosphlipids surrounding neutral lipids which is found in oil seeds for the storage of neutral lipids. This invention confirms that the oil bodies, structure consisting of proteins surrounding lipids, contribute to stability of lipids in the post-food processing and also found easy ways to fractionate and recover them. Further, in this invention the "oil body-like" means that part of the oil bodies is changed in the preparation like by extraction.

The invention is aimed at the oil body-like nanocapsule fraction or its dried form which contain oleosin-containing oil seeds, preferably soybean-derived, or betst, oleosin proteins over 10% of the the total proteins. To be more specific, the invention is aiming at the oil body-like nanocapsule fractions containing oleosin-protein-containing oil seeds, preferably soybean-derived, or best, oleosin proteins over 10% of the total proteins, the dried matrials, the shell-like nanocapsules wherein neutral lipids have been removed from the nanocapsule fraction, preferably the shell-like nanocapsules for the inclusion of hydrophobic substances. In practice it is aimed at the preparation of shell-like nanocapsules for the inclusion of more one lipid selected from rapeweed oil, fat-soluble vitamins and fish oil, etc.

The following allergenic proteins are known in soybean (Table 1). The invention is about the oil body-like nanocapsule fraction which does not contain at least Gly m Bd 30K of these allergenic protein, the Gly m Bd 30K protein-missing, oleosin protein-containing oil seeds, preferably soybean-derived seeds, or best, oleosin-protein whose content is over 10% of the total protein. It is about the dried oil body-like nanocapsule fraction free of neutral lipids which is made into shell-like nanocapsules, preferably the shell-like nanocapsules for the inclusion of hydrophobic substances, or more practically, the shell-like nanocapsules for the inclusion of more the one lipid selected from rapeseed oil, fat-soluble vitamins and fish oil, etc.

Table 1

Protein component (kDa)	Fraction	Dectection* frequencey(%)
70-68	7S (alpha subunit)	23.2
67-63	7S	18.8
55-52	7S	14.5
50-47	7S	13.0
45-43	7S (Beta-subunit)	10.1
41-40	7S	7.2
38-35	7S	7.2
35	11S (acidic subunit)	1.4
35-33	7S	15.9
31-29	Whey (high m.w. fraction)	4.3
30	7S (Gly m Bd 30K)	65.2
28	7S (Gly m Bd 30K)	23.2
21-18	Whey (low m.w. fraction)	7.2
20	2S (Knitz type trypsin inhibitor	2.9
17	2S	1.4
15-14	2S	2.9

* Detection frequency out of 69 patients who were soybean protein-positive.

Quoted from: Ogawa et al. Biosci. Biotec. Biochem., 57: 1030-1033 (1993).
Ogawa et al. J. nutr. Sci. Vitaminol., 37: 555-565 (1991).

Also, in this invention as the starting material oleosin-containing oil seeds are used. The seeds are soaked in water, ground or after grinding soaked in water to separate the insolubles and the suspension is prepared. The suspension is heated at temeprature above 60 °C, or after heating at temperature above 60 °C the insolubles are separated to get a suspension to prepare oil body-like nanocapsule fraction of high oleosin purity. It is characterized by centrifuging the solution after heating to fractinate and to enhance purity of oleosin in the fraction. In this case as the starting material an oleosin-containing oil seeds are used. The said material is soaked in water and then grind, or after grinding they are soaked in water to separate the insolubles from the suspension. The suspension is then heated at temperature above 60 °C, or heat first at temperature above 60 °C then the insolubles are separated to get a suspension. The suspension is then centrifuged to get oleosin-rich oil body-like nanocapsules.

The invention is also aiming at preparing shell-like nanocapsules, obtained by the said method, which are free of neutral lipids, or about the drying the oil body-like nanocapsules followed by removing neutral lipids to make shell-like nanocapsules.

Execution of the invention: As the starting material any oil seeds are acceptable so long as they contain oleosins, preferably soybean.

Oil body exists in soybean but when soybean is made into soy milk nobody knew what an oil body turns into. When soybean is ground and extracted by water most of the oil bodies bind with proteins and form lipid/protein complexes (diameter, about 380 nm). On the other hand, in the presence of 1M NaCl some oil bodies can be separated. Size of the separated oil bodies is 100-400 nm in diameter and are spherical which is about the same as in soybean. Soy milk is prepared by heating the water extract of soybean. By heating at 65-80 °C the lipid/protein complex is disrupted and at 80-90 °C oil bodies are separated. 65-80 °C is the denaturation temperature of beta-conglycinine and denaturation of this protein leads to disruption of the complex particles and at 80-90 separation of oil bodies occur. This fact indicates that components of oil body binds with glycinine and further through beta-conglycinine lipi/protein complex is formed. Separated oil body component(s) by heating contains lipids, phospholipids and oleosin protein. Such a change is illustrated in Fig. 1.

Also, when thermal denaturation characteristics of oleosin was studied with a heat analyzer (U.S. Parkin-Elmer Co. model Pyris-1) it was confirmed that its denaturation point was around 130 °C. Therefore, when soy milk is prepared by heating below 100 °C no denaturation occurs. It is difficult to conceive that in the ordinary preparation of soy milk big change occurs to oil bodies and they seem to still assume the same shape as before heating. Lipids in soy milk stay stably inside the shell of the body-like components and will not separate by long term storage or reheating. Further, the oil body-like component itself is stably dispersed in water for long period of time and will not flocculate. This was verified by the following experiment: A touch mixer was used oil body-like components were dispersed in 0.02% sodium azide solution (a preservative) so its concentration would bed the same as in soy milk. Change in turbidity of the solution was checked with a spectrophotometer at 600 nm. If oil bodies coalesced, turbidity would decline but no decline was observed even after one month.

Preparation of oil body-like component: When raw soy milk was gradually heated, around 75 °C giant particles of smaller specific gravity were formed in soy milk. When such giant particles were examined they were found to be the oil body-like component consisting primarily of oleosin protein. Besides, these giant particles float under mild centrifugation (6200 x g, 30 minutes) and recovered as a fraction which did not contain too much 7S, 11S and allergens. Fig. 2 shows the particle distribution of the lipid/protein complexes in soy milk observed by heating at 75 °C, using a light scattering type lase beam particle distribution counter (U.S. Coulter Co. type LS-230). At 75 °C for 10 minutes 6-10 micron diameter giant particles were formed. Fig. 3 shows change in particle distribution when soy milk was heated at 65-90 °C for five minutes each. The 0.38 and 1.6 micron particles seen in the unheated soy milk shifted to large particles with the rise of temp-

erature and when heated at 90 °C it was verified that they changed into the 0.38 micron lipid/protein complex particles and the 7S subunit/ 11S basic subunit complex particles of 0.07 micron diameter. When the heating was raised (or heated for longer time) the giant particles disappeared but the oil body-like component with oleosin protein as the parimary component almost free of 7S.11S proteins and allergenic proteins was recovered as the supernated fraction by centrifugation at 12,000 x g for 30 minutes. This means that high temperature heating is effective in enhancing the purity of the oil body component but the conditions for the recovery by centrifugation will somewhat get worse. On the other hand, low temperature heating somewhat reduce purity of the oil body component but it can be recovered under milder conditions. Also in either case almost 100% of oil body in soy milk was recovered as the floating fraction. Conditions for the formation of giant particles varied with temperature and time but the optimal conditions were 70-80 °C for 2-15 minutes. if low temperature was used, longer time is needed while at higher temperature shorter time is required. However, as mentioned before, to recover giant particles as the floating fraction it is not necessary to facilitate formation of giant particles but it can be recovered as the floating fraction by heating at temperature above 60 °C.

Now, preparation of the dried shell-like nanocapsules of oleosin-rich oil body will be explained.

(1) Raw soy milk is heated above 60 °C, preferably more than five minutes to separate the oil body component from the lipid/protein complex particles.
(2) The 7S and 11S proteins denature at 95 °C for five minutes. By centrifuging such a solution for fractionation it is possible to get oil body of high oleosin purity free of allergenic proteins, 7S protein and 11S protein (Fig. 4, lane 4). The particles are less than one micron in diameter and their center of distribution is around 380 nm. The denaturation temperature of oleosin is around 130 °C.
(3) The resultant oil body is almost free of other proteins and thus Gly m Bd 30K, known as the allergenic protein was eliminated.
(4) At 75-80 °C for five minutes the oil body can be separated without denaturing 11S.
(5) Lipid is removed from the resultant oil body and the resultant shell-like material can be used as nanocapsules to include rapeseed oil, fat-soluble vitamins, fish oil and other hydrophobic substances. Inclusion of hydrophobic substance is irreversible.

Expansion of the applications of the oil body production by-product: As the boproduct in the preparation of oil body-like component thereare separated soy proteins. They are separate from the floating fraction when the heated soy milk is centrifuged (the supernate and precipitate fractions). Such proteins are almost free of lipids. When soy milk

is heated at high temperature 7S and 11S undergo denaturation which will limit their applications (reduction of their utility). On the other hand, when the milk is heated at low temperature these proteins do not undergo big changes so after separation of the oil body-like component they can be used for various purposes as the separated soy proteins. Here, in either case various unpleasant flavor materials in soybean migrate with the oil body-like component as the floating fraction so the separated soy proteins could have better flavor.

Examples:

Details of the invention will be given in the examples. However, the invention is not limited to these examples.

Example 1 (Preparation of oil body-like component, #1):

(1) Forty gm of soybean seeds were washed and put into a 200 ml beaker. Water was added to 150 ml level for soaking overnight in a refrigerator (4 °C). Weight of the water-soaked soybean seeds was 81.8 gm.
(2) Water was added to the water-soaked soybean seeds to the total weight of 300 gm. It was ground in a mixer for two minutes. Small amount of defoamer was added and ground for another two minutes.
(3) Cotton was spread on a Buchner funnel and the said ground material was suction-filtered which gave the unheated soy milk. Volume of the soy milk was 230 ml.
(4) This unheated soy milk was transferred into a 500 ml conical flask and heated in boiling water. When it reach 75 °C it was heated at this temperature for 10 minutes, then cooled down to room temperature (about 25 °C) with cold water.
(5) The resultant soy milk (about 230 ml) was centrifuged at 6,200 x g for 30 minutes and the floating (cream) fraction was recovered. About 85% of soybean oil in the seeds stayed in this fraction so the recovery of the oil body-like component was about 85%.

Example 2 (Preparation of oil body-like component, #2):

Oil body-like nanocapsules were prepared as in Example 1 except that in step (4) heating was done at 95 °C, and in step (5) centrifugation was done at 25,000 x g for 30 minutes. By this procedure recovery of the oil body-like component was almost 100%. Here, the supernate and the precipitate fractions aside of the floating fraction from centrifugation will contain proteins almost free of lipids. As various unpleasant flavor materials in soybean will migrate to the floating fraction together with the oil body-like component

oleosin in Lanes 4 and 5 was respectively about 94$ and 40% of the oil body-like nanocalsule shell total protein. When 300 mg of soybean oil was added to 10 mg of the nanpcapsule shell of 94% oleosin content for dispersion in water and its emulsification stability was studied by change in turbidity it was found that like the oil body-like component before defatting, no decline in tubidity was observed even after a month.

Benefits: By this invention it is possible to get the oil body-like nanocapsules derived from oil seeds. The material can be used in the preparation of shell-like nanocapsules for the stabilization of hydrophobic substances. By the invention it is also possible to the hydrophobic substance-including nanocapsules with the switching of neutral lipids in the oil body. Oil body-like nanocapsules can be obtained free of the allergenic protein Gly m Bd 30K. The invention offers the process to prepare oil body-like nanocapsules by efficient separation and recovery of the oil body. Also, the process can offer preparation of protein byproducts from the oil seeds which have better flavor. It also offers a process to prepare shell-like nanocapsules from the oil body-like nanocapsules.

Brief Explanation of the Figures

Fig. 1: A diagram showing changes the lipid/protein complex particles in the water extract of soybean seeds undergo to become soy milk during heating.

Fig. 2: A diagram showing temporal change of soy milk particle size distribution during heating at 75 °C.

Fig. 3: A digital figure showing SDS gel electrophoresis of the separated components formed under different conditions.

the separated proteins had better flavor.

Example 3 (Preparation of nanocapsule shells):

(1) The oil body-like component (floating fraction) obtained from Example 1 or 2 was dispersed in water and drop by drop it was added to liquid nitrogen for quick freezing followed by lyophilization.
(2) The material was defatted with n-hexane to get nanocapsule shells.

Analysis of protein composition of nanocapsule shells by SDS gel electrophoresis:
Components formed and separated under various conditions were run through SDS gel electrophoresis (separation gel concentration, 12.5%). Results are shown in Fig. 4. Component in each lane of Fig. 4 is as follows:

Lane 1: The supernate fraction obtained from ultracentrifugation (156,000 x g, 30 minutes) of the soy milk heated at 95 °C for five minutes.
Lane 2: Oil body fraction obtained by treating soybean seeds with 0.1 M NaCl, 1% 2-mercaptoethanol (reductant) and base (pH 9) followed by ultracentrifugation (156,000 x g, 30 minutes).
Lane 3: Oil body-like component obtained by ultracentrifuging the unheated soy milk (156,000 x g, 30 minutes).
Lane 4: Oil body-like component obtained by centrifuging (156,000 x g, 30 minutes) heated (95 °C, five minutes) soy milk.
Lane 5: The floating fraction obtained by centrifuging (6,200 x g 30 minutes) heated soy milk (75 °C, five minutes).
Lane 6: The floating fraction obtained by centrifuging (6,200 x g, 30 minutes) the heated soy milk (75 °C, 10 minutes).
Lane 7: The floating fraction obtained by centrifuging (25,000 x g, 30 minutes) the heated soy milk (75 °C, 15 minutes).
Lane 8: Heated soy milk (95 °C, five minutes).

In Fig. 4 α',α,β, Acidic, basic, G30K, O24K and O18K denoted the following:
α', α,β: Each subunit of beta-conglycinine protein.
Acidic, basic: Acidic and basic subunits of glycinine protein.
G30K: Gly m Bd 30K protein.
O24K, O18K: Oleosin proteins.

As shown in Fig. 4 the defatted floating (cream) fraction is the oil body-like nanocapsule shell fraction of the soybean seeds containing oleosin proteins. Also, content of

Fig. 1

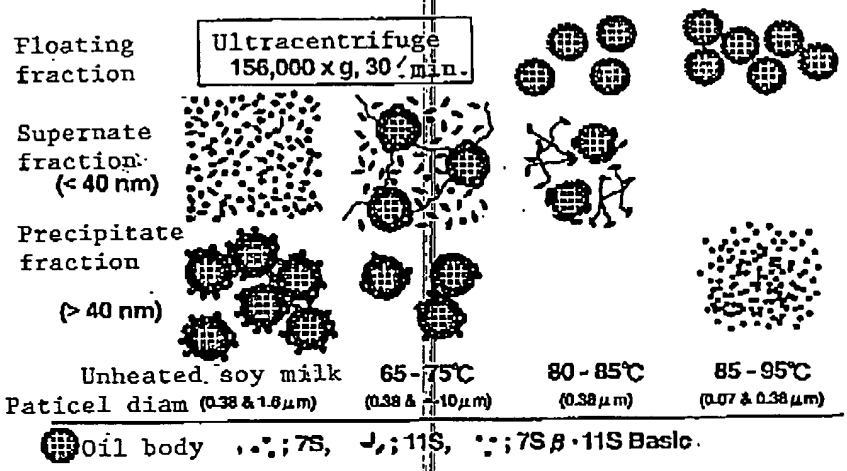

Floating fraction
Ultracentrifuge 156,000 x g, 30 min.
Supernate fraction (< 40 nm)
Precipitate fraction (> 40 nm)
Unheated soy milk
65 - 75℃
80 - 85℃
85 - 95℃
Paticel diam (0.38 & 1.6 μm)
(0.38 & 10 μm)
(0.38 μm)
(0.07 & 0.38 μm)
Oil body ; 7S, ; 11S, ; 7Sβ·11S Basic

Fig. 2

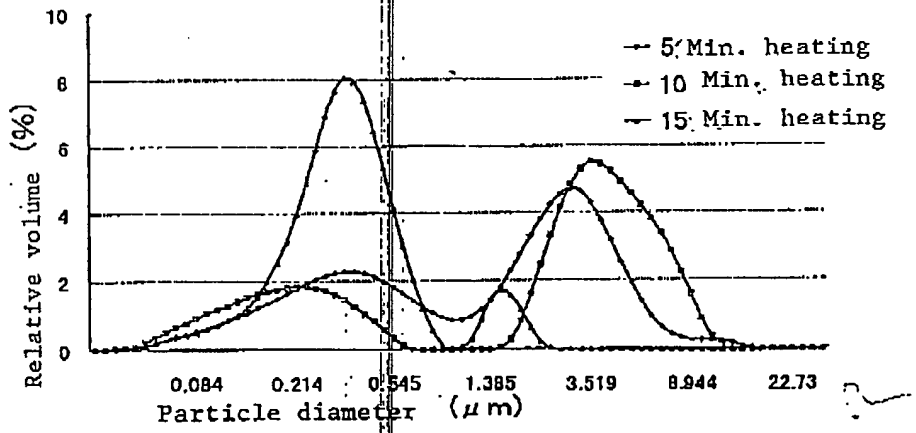

10
8
6
4
2
0
Relative volume (%)
5 Min. heating
10 Min. heating
15 Min. heating
0.084
0.214
0.545
1.385
3.519
8.944
22.73
Particle diameter (μm)

Fig. 3

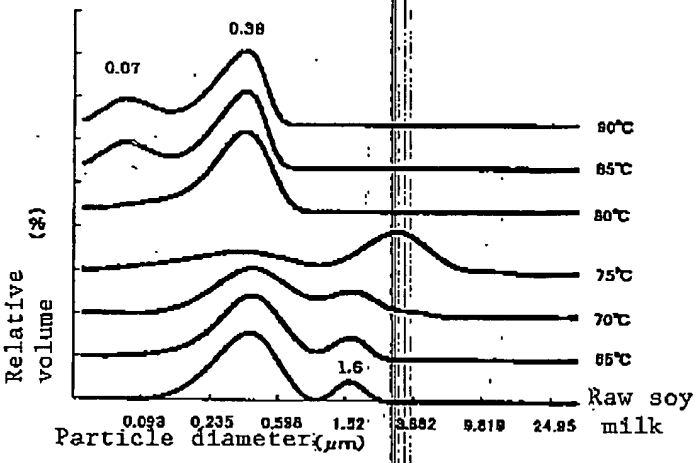

0.38
0.07
90℃
85℃
80℃
75℃
70℃
65℃
1.6
Raw soy milk
Relative volume (%)
0.093 0.235 0.588 1.52 3.682 9.819 24.95
Particle diameter (μm)

Fig. 4a

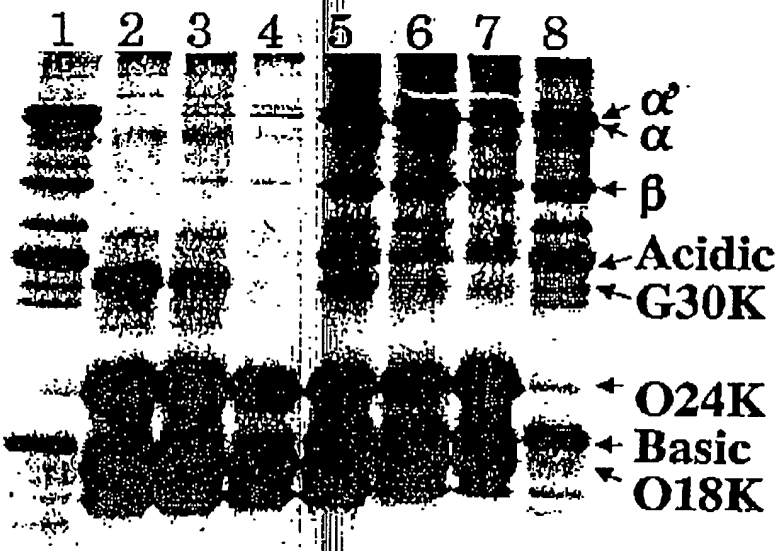

1 2 3 4 5 6 7 8
α'
α
β
Acidic
G30K
O24K
Basic
O18K

Fig. 4b